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## (54) Title: REGULATION OF NEURAL ENZYMES

## (57) Abstract

This invention relates to methods of regulating the effect of neural enzymes. It particularly relates to increasing the effective amount of the neural enzymes choline acetyltransferase (ChAT), glutamic acid decarboxylase (GAD) and nitric oxide synthetase (NOS) in the central nervous system (CNS) through the administration of GPE.

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## REGULATION OF NEURAL ENZYMES

This invention relates to methods of regulating the effect of neural enzymes. It particularly relates to increasing the effective amount of the neural enzymes choline acetyltransferase (ChAT), glutamic acid decarboxylase (GAD) and nitric oxide synthetase (NOS) in the central nervous system (CNS).

### BACKGROUND OF THE INVENTION

10 GPE is a tripeptide consisting of amino acids Gly-Pro-Glu. It and its dipeptide analogs Gly-Pro and Pro-Glu were first disclosed by Sara *et al* in EP 0366638. The suggestion made by Sara *et al* is that GPE has neuromodulatory properties (the capability of affecting the electrical properties of neurons). GPE has also been established as having neuroprotective properties and therefore having utility in the prevention or inhibition 15 of neural cell death (WO 95/17204).

To date however, there has been no teaching or suggestion of GPE or its analogs having any direct effect on the effective amount of neural enzymes present in the CNS. There has certainly been no suggestion of GPE having the ability to upregulate expression of 20 the neural enzymes, ChAT, GAD and NOS, and/or of their receptors.

#### CHOLINE ACETYLTRANSFERASE (ChAT)

ChAT is involved in the synthesis of the neurotransmitter acetyl choline. An ability to upregulate ChAT expression therefore has implications for neural, muscular and neuromuscular therapy and prophylaxis, including where the survival of neural cells 25 is not threatened.

GAD is involved in the synthesis of the important inhibitory neurotransmitter gamma amino butyric acid (GABA). An ability to upregulate GAD expression therefore has implications for neural therapy and prophylaxis.

30 NOS has multiple functions in the brain, including regulating blood flow, cell metabolism and cell survival. An ability to regulate NOS expression using GPE therefore has implications for neural therapy and prophylaxis, including where the survival of neural cells is not threatened.

It is the object of this invention to provide new approaches to neuronal therapy or prophylaxis which involve directly upregulating the expression of neural enzymes present in the CNS, or at least to provide the public with a useful choice of treatment for a neurological disorder by providing a stereospecific GAC, its analogs and/or its

## 5 SUMMARY OF THE INVENTION

In a first aspect, the invention provides a method of treatment of a patient suffering from or susceptible to a condition in which an increase in the amount of a neural enzyme selected from ChAT, NOS and GAD is desirable, which method comprises the 10 step of increasing the effective amount of GPE or an analog thereof within the CNS of said patient.

In a further aspect, the invention provides a method of increasing the amount of the neural enzyme ChAT in a patient for therapy or prophylaxis of a neurological disorder 15 or condition, said method comprising the step of increasing the effective amount of GPE or an analog thereof within the CNS of said patient.

In still a further first aspect, the invention provides a method of increasing the amount 20 of the neural enzyme GAD in a patient for therapy or prophylaxis of a neurological disorder or condition, said method comprising the step of increasing the effective amount of GPE or an analog thereof within the CNS of said patient.

In yet a further aspect, the invention provides a method of increasing the amount of the neural enzyme NOS in a patient for therapy or prophylaxis, said method comprising 25 the step of regulating the effective amount of GPE or an analog thereof within the CNS of said patient.

"Increasing the amount" of a neural enzyme is through upregulation of expression of the neural enzyme.

30

By "analog" it is meant the dipeptides Gly-Pro and Pro-Glu as well as any other small peptide which is capable of effectively binding to the receptors in the CNS GPE binds to and of inducing an equivalent upregulatory effect upon the expression of ChAT, GAD or NOS and/or their respective receptors.

Most preferably, it is the effective amount of GPE itself which is increased within the CNS of the patient. This can be effected by direct administration of GPE and indeed this is preferred. However, the administration of compounds which indirectly increase the effective amount of GPE (for example a prodrug which, within the patient is cleaved to release GPE) is in no way excluded.

The active compound (GPE or its analog) can be administered alone or, as is preferred, as part of a pharmaceutical composition. GPE is an active agent in the treatment of a number of diseases, including, but not limited to, GPE, Thalassemia, Thalassemia major, and

10. The composition can be administered to the patient peripherally (for example by a parenteral route such as injection into the peripheral circulation) or can be administered directly to the CNS. This latter route of administration can involve, for example, lateral cerebro-ventricular injection or a surgically inserted shunt into the lateral cerebro-ventricle of the brain of the patient.

15. *See also* *the section on public administration, political parties, and the press*.

Conveniently, the expression of ChAT and/or its receptors is upregulated through the administration of GPE or its analogs in the prophylaxis or therapy of one or more of the following:

## Peripheral neuropathies:

### Autonomic neuropathies: bradycardia, hypertension, and hypotension

**Memory loss, and the question of the reliability of our memory**

BRD 251: Neurodegeneration due to aging

Conveniently, the expression of GAD and/or its receptors is upregulated through the administration of GPE or its analogs in the prophylaxis or therapy of one or more of the following:

## 30 postasphyxial seizures:

convulsive disorders such as epilepsy; and

neurodegenerative diseases such as Huntington's.

Conveniently, the expression of NOS and/or its receptors is upregulated through the administration of GPE or its analogs in the prophylaxis or therapy of one or more of the following:

**subarachnoid haemorrhage;**

**5 transient ischemic attack;**

**stroke;**

**multinfarct dementia;**

**cerebral vasculitis; and**

**traumatic brain injury.**

10 In a further aspect, the invention also consists in the use of GPE or an analog thereof in the manufacture of a medicament for use in increasing the amount of ChAT, GAD or NOS present in the CNS.

## 15 BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is broadly as defined above. However, those persons skilled in the art will appreciate that it is not limited only to the above but that it also includes embodiments of which the following description provides examples. A better

20 understanding of the present invention will also be gained through reference to the accompanying drawings in which:

Figure 1 shows the number of ChAT-positive neurons following treatment with a control vehicle or with 3  $\mu$ g of GPE 2 hours after induced hypoxia;

25 Figure 2 shows the number of GAD-positive neurons following treatment with a control vehicle or with 3  $\mu$ g of GPE 2 hours after induced hypoxia;

Figure 3 shows the number of NOS-positive neurons following treatment with a control

30 vehicle or with 3  $\mu$ g of GPE 2 hours after induced hypoxia;

Figure 4 shows the effects of GPE on the number of GAD-positive neurons following

quinolinic acid-induced injury to the brain;

Figure 5 shows the effects of GPE on the number of NOS-positive neurons following 5 quinolinic acid-induced injury to the brain; and

Figure 6 shows the effects of GPE on the number of ChAT-positive neurons following 5 quinolinic acid-induced injury to the brain.

## DESCRIPTION OF THE INVENTION

### 1. Introduction

#### 1.1. Summary of the Invention

As indicated above, the present invention is broadly based upon the applicants 10 surprising finding that GPE and its analogs are capable of increasing the amount of 15 certain neural enzymes within the CNS. This increase, which is through upregulating 20 enzyme expression, is achieved through increasing the effective concentration or 25 amount of GPE or the analog in the CNS of a patient. The neural enzymes specifically 30 upregulated in effect are ChAT, GAD and NOS.

15

It is presently preferred by the applicants that GPE itself be used to increase the 20 amount of the neural enzyme. Most conveniently, this is effected through the direct 25 administration of GPE to the patient.

However, while this is presently preferred, there is no intention on the part of the 20 applicants to exclude administration of other forms of GPE. By way of example, the 25 effective amount of GPE in the CNS can be increased by administration of a prodrug 30 form of GPE which comprises GPE and a carrier, GPE and the carrier being joined by 35 a linkage which is susceptible to cleavage or digestion within the patient. Any suitable 40 linkage can be employed which will be cleaved or digested to release GPE following 45 administration.

25  
40  
45

It is further preferred that GPE be administered as part of a medicament or 20 pharmaceutical preparation. This can involve combination of GPE with any 25 pharmaceutically appropriate carrier, adjuvant or excipient. The selection of the 30 carrier, adjuvant or excipient will of course usually be dependent upon the route of 35 administration to be employed.

1.2. General Description of the Invention

The administration route can vary widely. An advantage of GPE is that it can be administered peripherally. This means that it need not be administered directly to the CNS of the patient in order to have effect in the CNS.

Any peripheral route of administration known in the art can be employed. These can include parenteral routes with injection into the peripheral circulation being a suitable example. However, alternative administration routes selected from oral, rectal, nasal, subcutaneous, inhalation, intraperitoneal or intramuscular can be employed.

Two of the most convenient administration routes will be by IV (eg. dissolved in 0.9% sodium chloride) or orally (in a capsule).

It will also be appreciated that it may on occasion be desirable to directly administer GPE to the CNS of the patient. Again, this can be achieved by any appropriate direct administration route. Examples include administration by lateral cerebro-ventricular injection or through a surgically inserted shunt into the lateral cerebro-ventricle of the brain of the patient. It will also be appreciated that it may be necessary to administer GPE to the CNS of the patient and to do this it will be necessary to calculate the amount to be administered. The calculation of the effective amount of GPE or its analogs to be administered will be routine to those persons skilled in this art. Needless to say, the final amount to be administered will be dependent upon the route of administration and upon the nature and form of the neurological disorder or condition which is to be treated. A suitable dose range may for example be between about 0.04 µg to 1,000 µg of GPE and/or analog per 100 kilograms of body weight where the dose is administered centrally.

GPE and its analogs can be obtained from a suitable commercial source. Alternatively, GPE and its analogs can be directly synthesised by conventional methods such as the Boc/Boc stepwise solid phase synthesis method of Merrifield *et al.* (*J. Amer. Chem. Soc.* **85** 2149-2156 (1963)). Alternatively, synthesis can involve the use of commercially available peptide synthesisers such as the Applied Biosystems model 430A.

The present invention will now be illustrated with reference to the following non-limiting examples. In this and the following examples, GPE is the acronym for Gammalamin.

**Example 1** The following example is not intended to be exhaustive with respect to the scope of the invention, and it will be apparent that many other modifications may be made.

The objective of these studies was to determine the effects of administering GPE on expression of ChAT in the presence or absence of CNS injury. The experiment involved treating the rats with a control vehicle or GPE 2 hours after a focal CNS injury. These rats had an hypoxic-ischemic injury to one cerebral hemisphere induced in a standard manner (ligation of the carotid artery). The degree and length of hypoxia, the ambient temperature and humidity were defined to standardise the degree of damage. The neuronal death is restricted to the side of the carotid ligation and is primarily in the hippocampus, dentate gyrus, striatum and lateral cortex of the ligated hemisphere. There is no neuronal loss in the contralateral hemisphere.

Specifically, nine pairs of adult wistar rats (280-320g) were prepared under halothane/O<sub>2</sub> anaesthesia. The right side carotid artery was ligated. A guide cannula was placed on the dura 7.5mm anterior from stereotaxic zero and 1.5mm from midline on the right. The rats were allowed to recover for 1 hour and were then placed in an incubator with humidity 90+/-5% and temperature 31+/-0.5°C for 1 hour before hypoxia. Oxygen concentration was reduced and maintained at 6+/-0.202% for 10 minutes. The rats were kept in the incubator for 2 hours after hypoxia and then treated either with 3ug GPE or vehicle (0.1M citrate buffer (pH6), diluted 10 times in 0.1% bovine serum albumin (in 0.1M phosphate buffered saline (PBS) (100ml each) (pH7.3)). A further 8 rats were used as normal controls. The rats were sacrificed using pentobarbital 3 days after hypoxic-ischemic injury. Brains were perfused with normal saline and 4% paraformaldehyde and fixed in perfusion fixative overnight.

Brains were stored in 25% sucrose in 0.1M PBS (pH7.4) until the tissue sank. Frozen coronal sections (30um) of striatum, globus pallidus and substantia nigra were cut using a microtome and stored in 0.1% sodium azide in 0.1M PBS at 4°C. Immunoreactivity for Choline acetyltransferase (ChAT) was established by staining using a free floating section method. Briefly, the antibodies were diluted in 1% goat serum. The sections were incubated in 0.2% triton in 0.1M PBS/triton at 4°C overnight before immunohistochemistry. The sections were pre-treated with 1% H<sub>2</sub>O<sub>2</sub> in 50% methanol for 20 minutes. The sections were then incubated with rabbit (Rb) anti-ChAT (1:5000) antibodies (the primary antibodies) in 4D on a shaker for two days. The sections were washed using PBS/triton (15 minutes x 3d) and then incubated with goat

anti-rabbit biotinylated secondary antibodies (1:1000) at room temperature overnight. The sections were washed and incubated in (ExtraAvidin TM Sigma 1:1000) for 3 hours with this solution. This was followed by streptavidin-allophycocyanin (1:1000) and followed by  $H_2O_2$  (0.01%) in 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma 100 mg/ml) solution (1:1000) for 10 minutes to give a brownish black (0.05%) reaction. These sections were mounted on chrome alum coated slides, dried, and then counterstained with hematoxylin (1:1000) for 5 minutes. The slides were then dehydrated and covered.

The striatal neurons in both hemispheres which showed specific immunoreactivities corresponding to ChAT were counted using a light microscope and a 1 mm 2x1000 grid. The size of the striatal region used for the count was measured using an image analyser. The total counts of neurons/mm<sup>2</sup> were compared between the GPE and vehicle treated group. Data were analysed with paired t-test and presented as mean  $\pm$  sem. Results are presented in Figure 1.

10 This figure shows that the number of ChAT immunopositive neurons increased in both the right and left (uninjured) sides. This clearly indicates that administration of GPE

15 is effective to upregulate ChAT expression.

100% EPO

This figure shows that the number of ChAT immunopositive neurons increased in both

15 the right and left (uninjured) sides. This clearly indicates that administration of GPE

is effective to upregulate ChAT expression.

100% EPO

## Example 2

100% EPO

20 The objective of these studies was to determine the effects of administering GPE on expression of GAD in the presence or absence of CNS injury. The experiment involved

15 treating the rats with a control vehicle or GPE 2 hours after a focal CNS injury. These

20 rats had an hypoxic-ischemic injury to one cerebral hemisphere induced in a standard

25 manner (ligation of the carotid artery). The degree and length of hypoxia, the ambient

temperature and humidity were defined to standardise the degree of damage. The

30 neuronal death is restricted to the side of the carotid ligation and is primarily in the

hippocampus, dentate gyrus, striatum and lateral cortex of the ligated hemisphere.

There is no neuronal loss in the contralateral hemisphere.

100% EPO

30 Specifically, nine pairs of adult wistar rats (280-320g) were prepared under

halothane/O<sub>2</sub> anaesthesia. The right side carotid artery was ligated. A guide cannula

35 was placed on the dura 7.5mm anterior from stereotaxic zero and 1.5mm from midline

40 on the right. The rats were allowed to recover for 1 hour and were then placed in an

45 incubator with humidity 90+-5% and temperature 31+-0.5°C for 1 hour before

hypoxia. Oxygen concentration was reduced and maintained at 6+/-0.202% for 10 minutes. The rats were kept in the incubator for 2 hours after hypoxia and then treated either with 3ug GPE or vehicle alone (0.1M citrate buffer [pH6], diluted 10 times in 0.1% bovine serum albumin in 0.1M phosphate buffered saline [PBS] [pH7.3]).

5 5 further 6 rats were used as normal controls. The rats were sacrificed

using pentobarbital 3 days after hypoxic-schematic injury. Brains were perfused with normal saline and 4% paraformaldehyde and fixed in perfusion fixative overnight.

10 Brains were stored in 25% sucrose in 0.1M PBS (pH7.4) until the tissue sank. Frozen

coronal sections (30um) of striatum, globus pallidus and substantia nigra were cut

15 using a microtome and stored in 0.1% sodium azide in 0.1M PBS at 4°C.

Immunoreactivity for GAD was established by staining using a free floating section

method. Briefly, the antibodies were diluted in 1% goat serum. The sections were

incubated in 0.2% triton in 0.1M PBS/triton at 4°C overnight before

immunohistochemistry. The sections were pre-treated with 1%  $H_2O_2$  in 50% methanol

20 for 20 minutes. The sections were then incubated with rabbit (Rb) anti-GAD (1:5000)

antibodies (the primary antibodies) in 4D on a shaker for two days. The sections were

washed using PBS/triton (15 minutes x 3d) and then incubated with goat anti-rabbit

25 biotinylated secondary antibodies (1:1000) at room temperature overnight. The

sections were washed and incubated in (ExtrAvidin TM Sigma 1:1000) for 3 hours and

30 followed by  $H_2O_2$  (0.01%) in 3,3-diaminobenzidine tetrahydrochloride (DAB) 0.05%

reaction. These sections were mounted on chrome alum coated slides, dried,

dehydrated and covered.

The striatal neurons in both hemispheres which showed specific immunoreactivities

35 corresponding to GAD were counted using a light microscope and a 1mm 2x1000 grid.

The size of the striatal region used for the count was measured using an image

30 analyser. The total counts of neurons/mm<sup>2</sup> were compared between the GPE and

vehicle treated group. Data were analysed with paired t-test and presented as mean

+- sem. Results are presented in Figure 2.

30

This figure shows that the number of GAD immunopositive neurons increased in the right side whereas Parvalbumin (a marker for the same cell type) was not increased. Showing that GAD expression was upregulated in the surviving cells (\*p<0.05).

**Example 3**

Studies in adult rats have shown that GPE can reduce neuronal death following a focal hypoxic-ischemic event. A project to extend this finding to the CNS is described below.

The objective of these studies was to determine the effects of administering GPE on expression of NOS in the presence or absence of CNS injury. The experiment involved

5 treating the rats with a control vehicle or GPE 2 hours after a focal CNS injury. These rats had an hypoxic-ischemic injury to one cerebral hemisphere induced in a standard manner (ligation of the carotid artery). The degree and length of hypoxia, the ambient temperature and humidity were defined to standardise the degree of damage. The neuronal death is restricted to the side of the carotid ligation and is primarily in the 10 hippocampus, dentate gyrus, striatum and lateral cortex of the ligated hemisphere.

There is no neuronal loss in the contralateral hemisphere.

Specifically, nine pairs of adult wistar rats (280-320g) were prepared under halothane/O<sub>2</sub> anaesthesia. The right side carotid artery was ligated. A guide cannula was placed on the dura 7.5mm anterior from stereotaxic zero and 1.5mm from midline on the right. The rats were allowed to recover for 1 hour and were then placed in an incubator with humidity 90+/-5% and temperature 31+/-0.5°C for 1 hour before hypoxia. Oxygen concentration was reduced and maintained at 6+/-0.202% for 10 minutes. The rats were kept in the incubator for 2 hours after hypoxia and then 20 treated either with 3ug GPE or vehicle alone (0.1M citrate buffer (pH6)) diluted 10 times in 0.1% bovine serum albumin in 0.1M phosphate buffered saline (PBS) (pH7.3). A further 6 rats were used as normal controls. The rats were sacrificed using pentobarbital 3 days after hypoxic-ischemic injury. Brains were perfused with normal saline and 4% paraformaldehyde and fixed in perfusion fixative overnight. 25 Brains were stored in 25% sucrose in 0.1M PBS (pH7.4) until the tissue sank. Frozen coronal sections (30um) of striatum, globus pallidus and substantia nigra were cut using a microtome and stored in 0.1% sodium azide in 0.1M PBS at 4°C. Immunoreactivity for neuronal nitric oxide synthetase (NOS) was established by staining using a free floating section method. Briefly, the antibodies were diluted in 30% 1% goat serum. The sections were incubated in 0.2% triton in 0.1M PBS/triton at 4°C overnight before immunohistochemistry. The sections were pre-treated with 1% H<sub>2</sub>O<sub>2</sub> and 1% BSA in 50% methanol for 20 minutes. The sections were then incubated with rabbit (Rb) anti-NOS (1:3000) antibodies (the primary antibodies) in 4D on a shaker for two days. The sections were washed using PBS/triton (15 minutes x 3d) and then incubated with

goat anti-rabbit biotinylated secondary antibodies (1:1000) at room temperature overnight. The sections were washed and incubated in (ExtraAvidin TM Sigma 1:1000) for 3 hours and followed by  $H_2O_2$  (0.01%) in 3,3-diaminobenzidine tetrahydrochloride (DAB, 0.05%) reaction. These sections were mounted on chrome alum coated slides, dried, dehydrated and covered.

The striatal neurons in both hemispheres which showed specific immunoreactivities corresponding to NOS were counted using a light microscope and a 1mm 2x1000 grid. The size of the striatal region used for the count was measured using an image analyser. The total counts of neurons/mm<sup>2</sup> were compared between the GPE and vehicle treated group. Data were analysed with paired t-test and presented as mean +/- sem. Results are presented in Figure 3.

This figure shows that the number of NOS immunopositive neurons increased in both the right and left (uninjured) sides (#p=0.072, \*p=0.008). This clearly indicates that the administration of GPE is effective to upregulate NOS expression. The data are as follows: right side: 15.10 +/- 1.0, uninjured side: 22.1 +/- 0.6, glomerular side: 15.10 +/- 1.0, left side: 15.10 +/- 1.0. **Example 4** For the first few examples and this one, the results are presented here for simplicity, right and left hemisphere counts are given in parentheses.

The objective of this study was to determine the effect of GPE administration on the expression of GAD, NOS and ChAT in the presence or absence of injury to the striatum induced by quinolinic acid. When injected into the striatum in experimental animals quinolinic acid produces lesions arising from the loss of striatopallidal and striatonigral GABAergic projection neurons, but spares the axons in the striatum (see 25 (Science, 219, 316-318 (1983))). Rats were either treated with a control vehicle or GPE 2 hours after administration of quinolinic acid (30  $\mu$ g equivalent to 100 mg/kg).

#### **Materials and Methods**

A total of 14 adult male Wistar rats (280-310g) were used in this investigation. Eight rats were anaesthetized using halothane/O<sub>2</sub> anaesthesia. An injection of quinolinic acid (78 units, Sigma, dissolved in 4M NaOH) was stereotactically placed in the dorsal region of the right striatum 0.8mm anterior to bregma, 2.5mm lateral to the midline and 4.0mm ventral to the pial surface. 2 hours later an injection of 3 $\mu$ g GPE or vehicle alone (0.1M citrate buffer [pH6], diluted 10 times in 0.1% bovine serum albumin in

0.1M phosphate buffered saline (PBS (pH7.3)) was administered into the right lateral ventricle via a guide cannula 7.5mm anterior from stereotaxic zero, 1.5mm from the midline on the right, and vertical 3mm. Six rats were used as normal controls.

5 The rats were sacrificed using pentobarbitol 3 days after the quinolinic acid induced striatal injury. Brains were perfused with 10% buffered formalin (pH7) and processed for immunoreactivity for GAD, NOS which are found in GABAergic interneurons in the striatum and ChAT, which synthesizes acetylcholine and is found in cholinergic neurons in the striatum. Brains were stored in 25% sucrose in 0.1M PBS (pH7.4) 10 until the tissue sank. Frozen coronal sections (30 $\mu$ m) of the striatum, globus pallidus and substantia nigra were cut using a microtome and stored in 0.1% sodium azide in 0.1M PBS at 4°C. Immunoreactivity for GAD, NOS and ChAT was established by staining using a free floating method. Briefly, the antibodies were diluted in 1% goat serum. The sections were incubated in 0.2% triton in 0.1M PBS/triton at 4°C overnight 15 before immunohistochemistry. The sections were pre-treated with 1% H<sub>2</sub>O<sub>2</sub> in 50% methanol for 20 minutes. The sections were then incubated either with rabbit anti-GAD (1:5000), rabbit anti-NOS (1:3000) or rabbit anti-ChAT (1:5000) in 4D on a shaker for two days. The sections were washed using PBS/triton (15 minutes x 3) and then incubated with goat anti-rabbit biotinylated secondary antibodies (1:1000, 20, Amersham) at room temperature overnight. Sections were washed and incubated in 25 (ExtrAvidin™, 1:1000, Sigma) for 3 hours and then reacted in 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.01% H<sub>2</sub>O<sub>2</sub> to produce a brown reaction product. These sections were mounted on chrome alum coated slides, dried, dehydrated and covered.

30 The striatal neurons in both hemispheres which showed specific immunoreactivities corresponding to GAD, NOS and ChAT were counted using a light microscope and a 1mm 2x 1000 grid. The size of the striatal region used for the count was measured using an image analyser (Mocha image analysis software). The total counts of 35 neurons/mm<sup>2</sup> were compared between the GPE and vehicle treated group. The data has not been analysed for statistical significance. Tissue from one of the GPE treated animals was unable to be counted. These results are presented in Figures 4-6.

10 **Results** In the present invention, GPE (2,6-diquinolyl pyridine) increases the levels of GAD, NOS and ChAT.

15 **Figure 4** shows a loss of GAD immunoreactivity in the striatum on both the left (non-injured) and right sides of the brain following injury. GPE induced an upregulation of GAD expression on both sides of the brain.

20 **Figure 5** shows a loss of NOS activity in the striatum after injury on both the left and right sides of the brain. GPE induced an upregulation of enzyme expression on both sides of the brain, restoring the enzyme levels to normal on the left (non-injured) side of the brain.

25 **Figure 6** shows a loss of ChAT immunoreactivity after injury on both sides of the brain. GPE treatment upregulated the levels of ChAT to above normal on the right (injured) side of the brain.

### 30 **Conclusions**

These results demonstrate the ability of GPE to regulate the expression of GAD, NOS and ChAT in the CNS.

35 Furthermore, the results indicate that GAD and NOS are regulated in the presence and absence of the quinolonic acid induced neural injury. This clearly demonstrates the effect of GPE in upregulating GAD and NOS expression independent of a response to neural damage or a threat to neural cell survival.

40 The results also show that ChAT can be upregulated by GPE in the presence of quinolonic acid induced neural injury.

### 45 **INDUSTRIAL APPLICATION**

50 The experimental results demonstrate the ability of GPE to increase the amount of the neural enzymes ChAT, GAD and NOS in the CNS through a direct increase in enzyme expression. Further, the results indicate that expression of both ChAT and NOS is upregulated both in the presence and absence of neural injury. This clearly represents

that the effect of GPE in upregulating expression of these enzymes is independent of a response to neural damage or a threat to neural cell survival.

an upregulation of GPE response to neural damage is limited to their native cell type. These findings make GPE and its analogs applicable in treating a number of 5 neurological disorders or conditions, either therapeutically or prophylactically. Indeed, it will be apparent to those persons skilled in the art that GPE and its analogs can be employed at any time where a patient would benefit from an increase in the expression

of ChAT, GAD or NOS within the CNS. Neurological disorders or conditions which would benefit from this include, but are not limited to the following:

10

motor neuron disease, Alzheimers disease, muscular dystrophy, peripheral neuropathies, autonomic neuropathies, memory loss, aging and other forms of neurodegeneration (ChAT);

15

postasphyxial seizures, epilepsy and other convulsive disorders, neurodegenerative diseases such as Huntingtons, plus the immediate post acute phase following head trauma, stroke, and other forms of hypoxic ischemic brain injury (GAD); and

20

subarachnoid haemorrhage, transient ischemic attack, stroke, multifarct dementia, cerebral vasculitis and traumatic brain injury plus the immediate post acute phase following head trauma, stroke and other forms of hypoxic ischemic brain injury.

25

It will be appreciated that although the present invention is described above with reference to certain specific embodiments, the description provided is exemplary only and that the invention is limited only by the lawful scope of the appended claims.

**CLAIMS**

1. A method of treatment of a patient suffering from or susceptible to a condition

in which an increase in the amount of a neural enzyme selected from ChAT, 5 NOS and GAD is desirable, which method comprises the step of increasing the effective amount of the GPE or an analog thereof within the CNS of said patient.

2. A method of treatment as claimed in claim 1, wherein the concentration of GPE or an analog thereof is increased by administering to said patient an effective 10 amount of GPE or said analog of GPE or of a prodrug thereof.

3. A method of treatment as claimed in claim 2, wherein the concentration of GPE is increased in the CNS by direct administration of GPE.

15 4. A method according to any one of claims 1 to 3 which is prophylactic.

5. A method according to any one of claims 1 to 3 which is therapeutic.

6. A method according to any one of claims 1 to 5 wherein the condition is one in 20 which an increase in the amount of ChAT is desirable.

7. A method according to claim 6 wherein the condition is selected from Motor 25 Neuron disease, Alzheimer's disease, muscular dystrophy, peripheral neuropathies, autonomic neuropathies, memory loss and neurodegeneration due to aging.

8. A method according to any one of claims 1 to 5 wherein the condition is one in 30 which an increase in the amount of GAD is desirable.

9. A method according to claim 8 wherein said condition is selected from postasphyxial seizures, convulsive disorders, neurodegenerative diseases and hypoxic ischemic brain injury.

10. A method according to any one of claims 1 to 5 wherein the condition is one in which an increase in the amount of NOS is desirable.

11. A method according to claim 10 wherein said condition is selected from subarachnoid haemorrhage, transient ischemic attack, stroke, multisarct dementia, cerebral vasculitis and traumatic brain injury.

12. A method of upregulating the expression of the neural enzyme ChAT in a patient for therapy or prophylaxis of a neurological disorder or condition, said method comprising the step of increasing the effective amount of GPE or an analog thereof within the CNS of said patient.

13. A method of upregulating the expression of the neural enzyme GAD in a patient for therapy or prophylaxis of a neurological disorder or condition, said method comprising the step of increasing the effective amount of GPE or an analog thereof within the CNS of said patient.

14. A method of upregulating the expression of the neural enzyme NOS in a patient for therapy or prophylaxis of a neurological disorder or condition, said method comprising the step of regulating the effective amount of GPE or an analog thereof within the CNS of said patient.

15. A method according to any one of claims 11 to 13 which comprises direct administration of GPE, an analog thereof or a prodrug thereof to the CNS of said patient.

16. The use of GPE or an analog thereof in the preparation of a medicament for use in increasing the amount of a neural enzyme selected from ChAT, GAD and NOS within the CNS of a patient for therapeutic or prophylactic purposes.

17. The use of claim 16 wherein the medicament is for use in increasing the expression of ChAT in the CNS of a patient.

18. The use of claim 16 wherein the medicament is for use in increasing the expression of GAD in the CNS of a patient.

19. The use of claim 16 wherein the medicament is for use in increasing the  
5 expression of NOS in the CNS of a patient.

Levante (1960) en el que se establece que el efecto de la actividad humana en el ecosistema es de tipo degradante y no degradante, dependiendo de la actividad humana.

COLLECTIVE PRACTICE, POLITICAL

The 2010 study of Hart et al. (2010) reported 100% of patients with glioma to be positive for *EGFR* mutations.

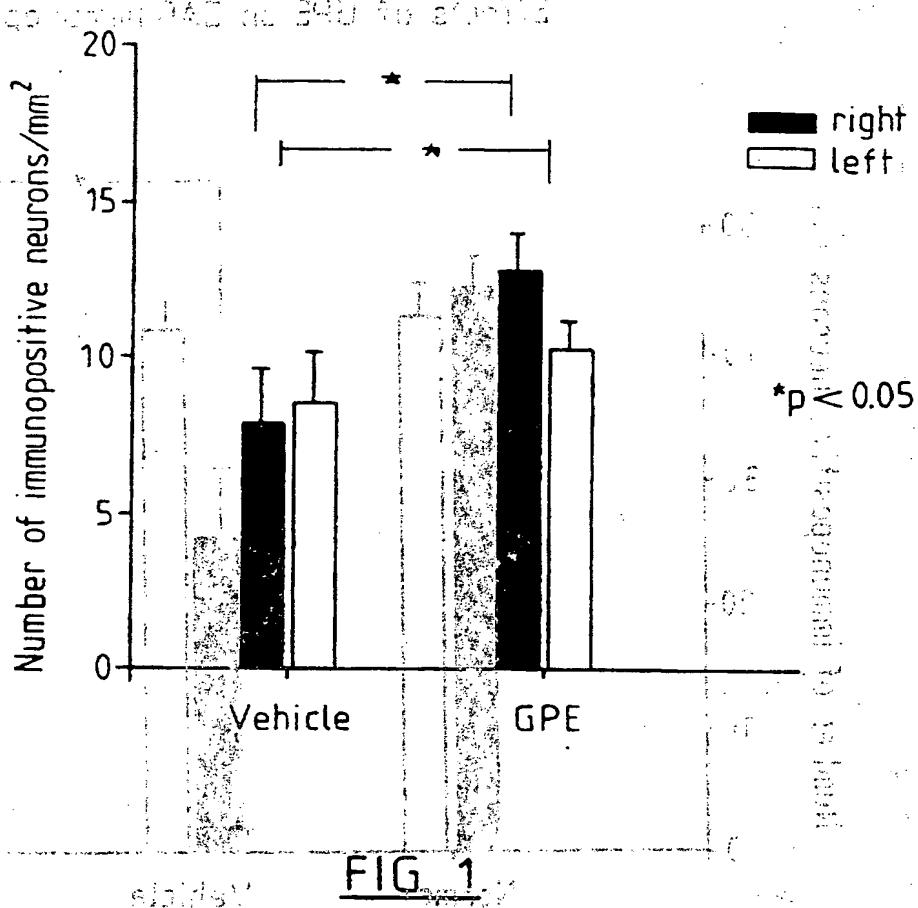
ANSWER: **1. 1000** (1000 is the only number that is a multiple of 1000 and is also a factor of 1000.)

the 1970s, the number of people in the U.S. with incomes below the poverty line fell from 13.5% to 11.1% of the population. The number of people in poverty fell from 36.4 million to 33.3 million.

新編 五十年文庫 1972年 1月 10日

1/6

## Effects of GPE on ChAT immunopositive neurons



## Effects of GPE on GAD immunopositive neurons

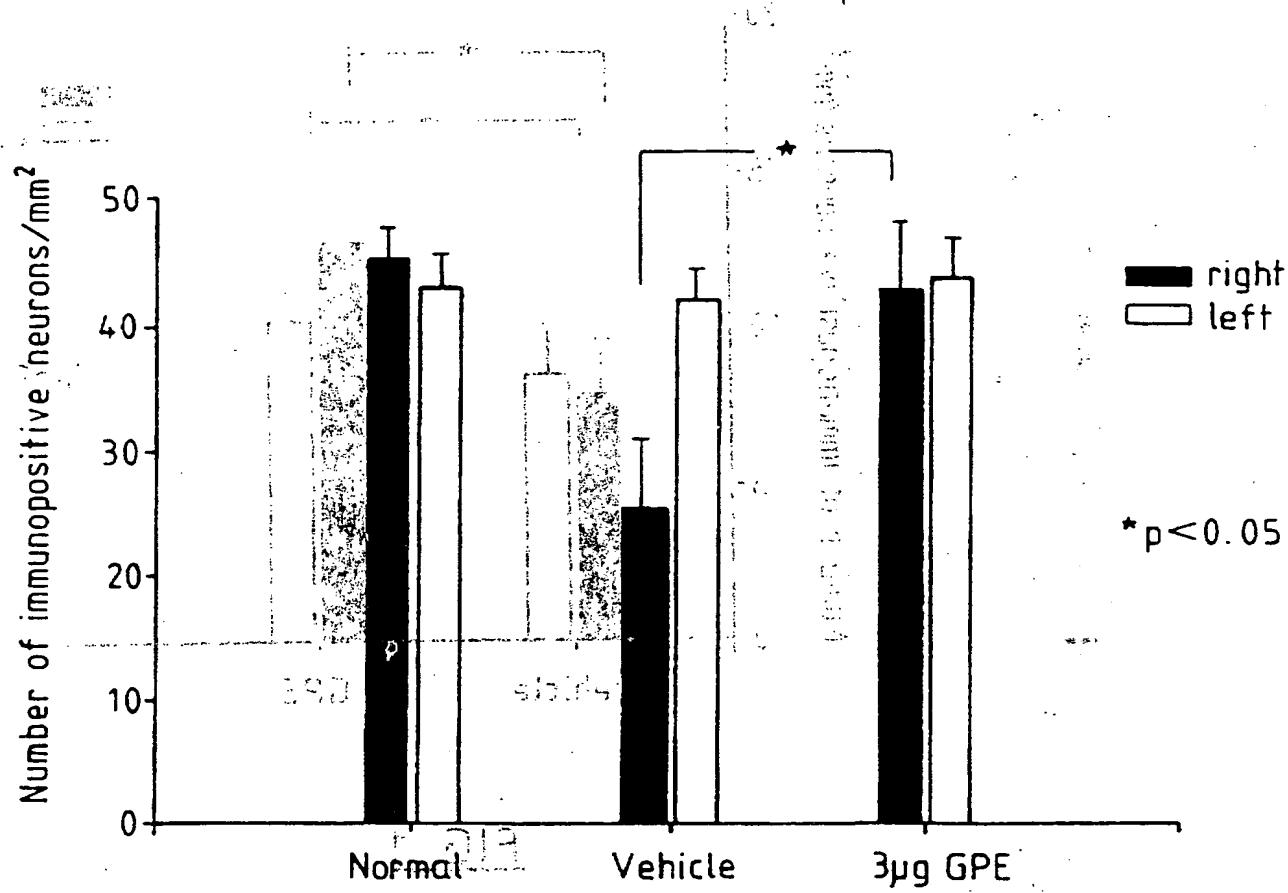


FIG. 2

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## Effects of GPE on NOS immunopositive neurons

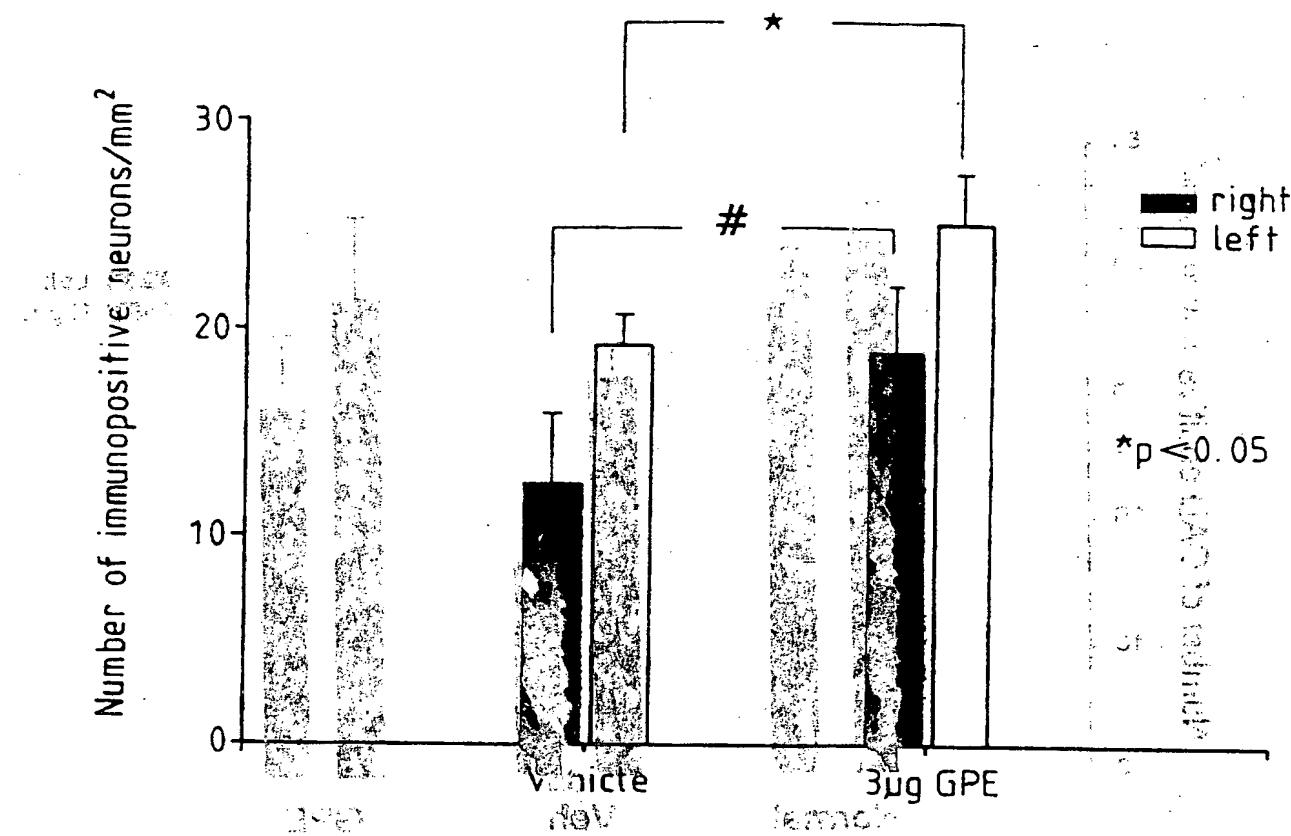


FIG. 3

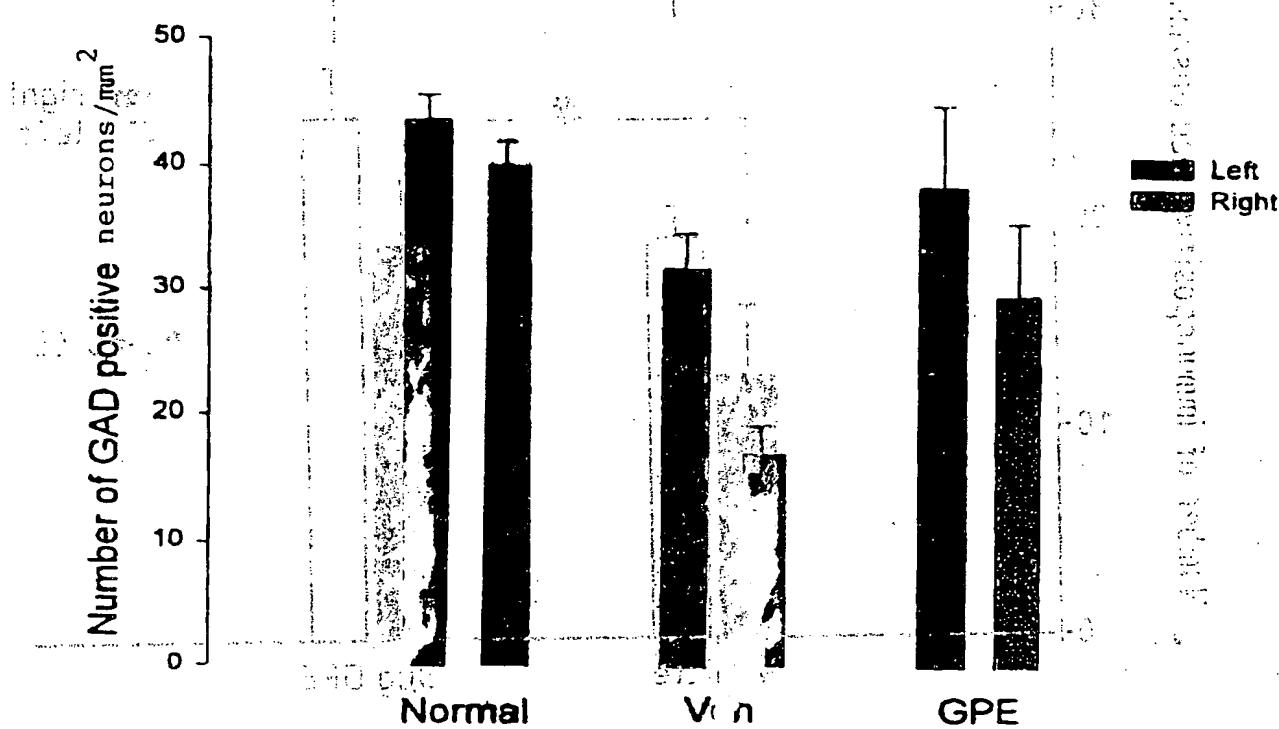


FIG. 4

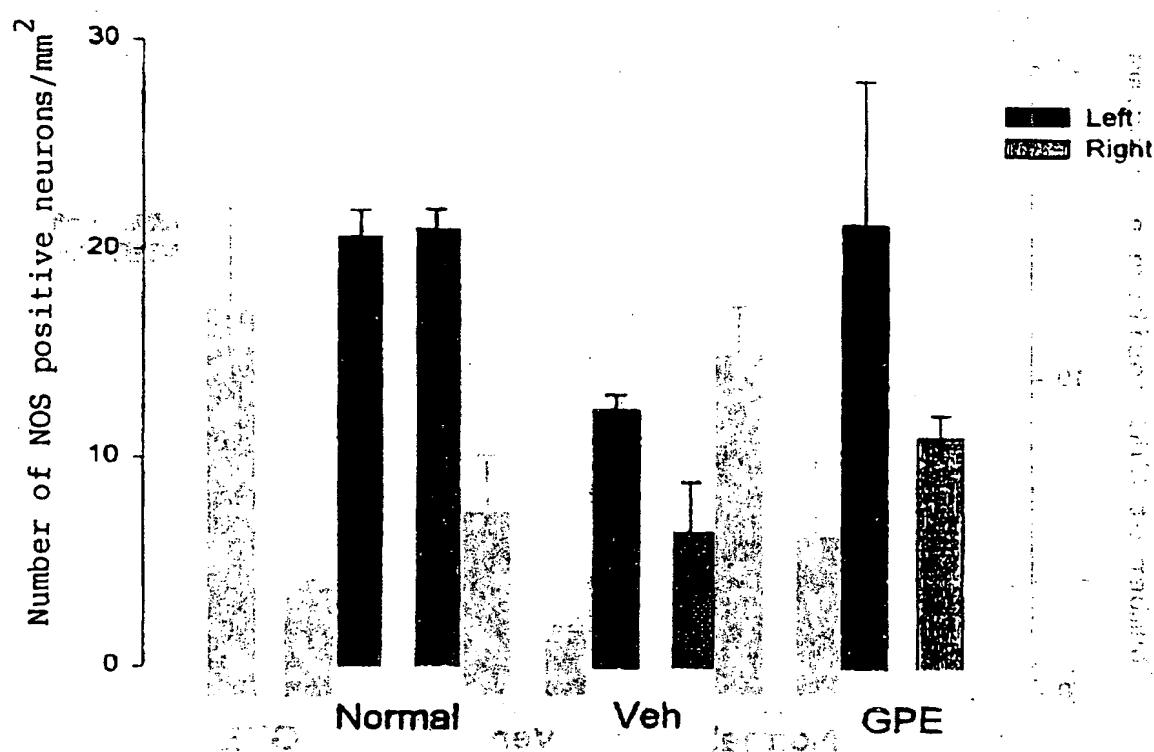


FIG. 5

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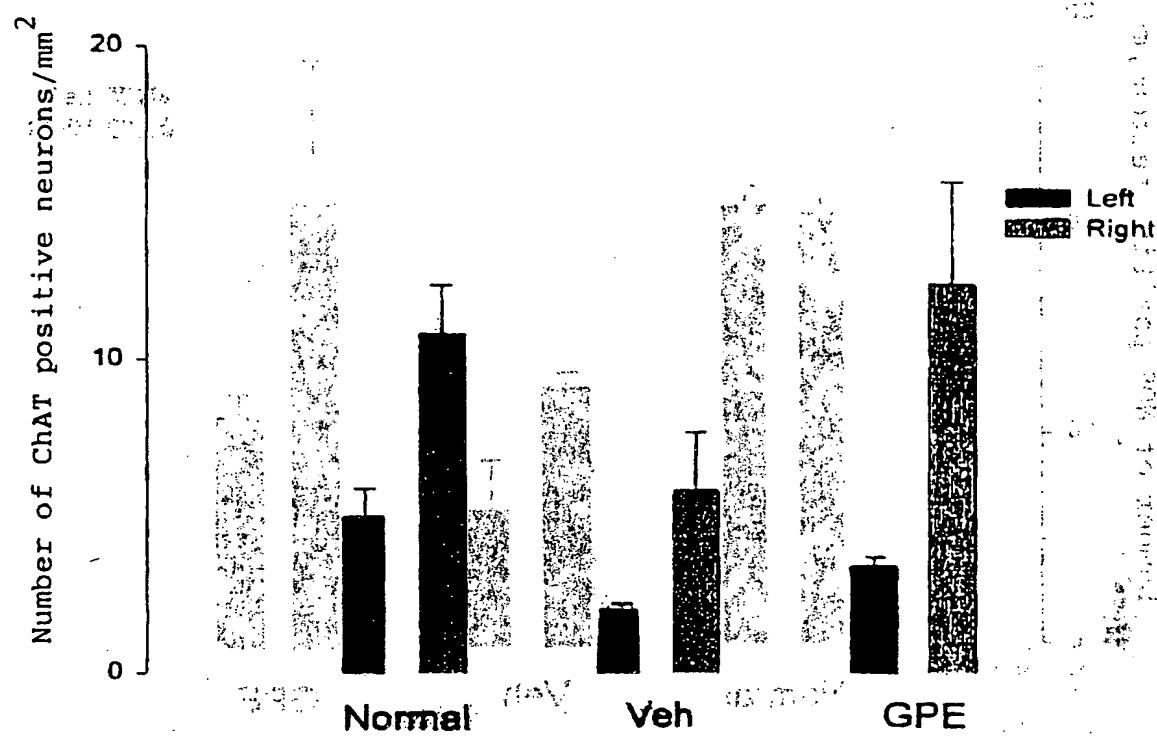


FIG. 6

SUBSTITUTE SHEET (RULE 26)

# INTERNATIONAL SEARCH REPORT

PATENT WORKS

International Application No.  
PCT/NZ 97/00132

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup> A61K 38/05, 38/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61K 38/05, 38/06

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
DERWENT WPAT-[GLY() PRO()GLU OR GPE] AND A61K37/IC. OR 38/IC. OR 39/IC , CHEMICAL ABSTRACTS-[GLY-PRO-GLU], [32302-76-4] , MEDLINE - GPE, STN, SEQUENCE Search

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 366638 (KABIGEN AB) Published 2 May 1990, See column 1, lines 34-49 in particular	1-8, 10-19
X	WO, A1:95/17204 (AUCKLAND UNISERVICES LIMITED) Published 29 June 1995, claims and abstracts	1-6, 8, 10, 12-19

Further documents are listed in the continuation of Box C  See patent family annex

"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier document but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 5 January 1998	Date of mailing of the international search report 19 JAN 1998
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer S.Chandra Telephone No.: (02) 6283 2264

**INTERNATIONAL SEARCH REPORT**

SEARCHED

International Application No.

PCT/NZ 97/00132

<b>C (Continuation)</b>		<b>DOCUMENTS CONSIDERED TO BE RELEVANT</b>	
<b>Category*</b>		<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to claim No.</b>
X		SARA, V.R. et al. (1989) Identification of Gly-Pro-Glu(GPE), the aminoterminal tripeptide of insulin-like growth factor 1 which is truncated in brain, as a novel neuroactive peptide, Biochemical and biophysical research Communications, Volume 165, No. 2, 15 December 1989, pages 766-771	1-6, 8, 10, 12-19
X		NILSSON-HAKANSSON, L. et al. (1993) Effects of IGF-1, truncated IGF-1 and the tripeptide Gly-Pro-Glu on acetylcholine release from parietal cortex of rat brain, NeuroReport, Volume 4, No. 9, 6 August 1993, pages 1111-1114.	1-6, 8, 10, 12-19
CD A B C D		SARA, V.R. et al. (1993) The Biological Role of Truncated Insulin-like Growth Factor-1 and the Tripeptide GPE in the Central Nervous system, Annals of the New York Academy of Sciences, Volume 692, pages 183-191.	1-19
CD A		SARA, V.R. et al. (1991) Neuroactive products of IGF-1 and IGF-2 gene expression in the CNS, Molecular Biology and Physiology of Insulin and Insulin like Growth Factors, Edited by Raizada, M.K. and LeRoith, D., Plenum Press, New York, 1991, pages 439-448.	1-19
CD A		GLUCKMAN, P.D. et al. (1994) The role of IGF-1 in the response to organ injury - studies in the central nervous system, The insulin-like growth factors and their regulatory proteins, pages 427-434	1-19
A		AU, A1, 38084/93 (RUSSIAN-AMERICAN INSTITUTE FOR NEW FRUG DEVELOPMENT) PUBLISHED 28 October 1993, See the abstract and claims	1-19

**INTERNATIONAL SEARCH REPORT**  
Information on patent family members

**International Application No.**  
**PCT/NZ 97/00132**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
EP	366638	CA	2001498	JP	2250895	SE	8803847
WO	95/17204	AU	13281/95	CA	2178711	EP	735894
				JP	9509404		
AU	93/38084	US	5439930	WO	93/21216		

**END OF ANNEX**